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The negligible effects of the antifungal natamycin on cholesterol-dipalmitoyl phosphatidylcholine monolayers may explain its low oral and topical toxicity for mammals

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ABSTRACT

Natamycin is an effective, broad spectrum antifungal with no reported resistance, in contrast to most antimicrobials. It also exhibits reduced (oral and topical) toxicity to humans, which is probably associated with the lack of effects on mammalian cell membranes. In this paper we employ Langmuir monolayers to mimic a cell membrane, whose properties are interrogated with various techniques. We found that natamycin has negligible effects on Langmuir monolayers of dipalmitoyl phosphatidylcholine (DPPC), but it strongly affects cholesterol monolayers. Natamycin causes the surface pressure isotherm of a cholesterol monolayer to expand even at high surface pressures since it penetrates into the hydrophobic chains. It also reduces the compressibility modulus, probably because natamycin disturbs the organization of the cholesterol molecules, as inferred with polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS). In mixed cholesterol/DPPC monolayers, strong effects from natamycin were only observed when the cholesterol concentration was 50 mol% or higher, well above its concentration in a mammalian cell membrane. For a sterol concentration that mimics a real cell membrane in mammals, i.e. with 25 mol% of cholesterol, the effects were negligible, which may explain why natamycin has low toxicity when ingested and/or employed to treat superficial fungal infections.

1. Introduction

Diseases caused by opportunistic fungal infections have become a major health problem owing to the increasing number of individuals whose immune system has been compromised by HIV infection or by administration of immunosuppressive drugs during cancer treatment and organ transplants [1,2]. This has motivated the development of new antifungal drugs, including polyene antibiotics [3], that are natural drugs typically produced by *Streptomyces* spp. [4]. Examples of these polyenes are amphotericin B, nystatin and natamycin, which are effective antifungals with no reported resistance since their discovery 50 years ago, in contrast to most antimicrobials. The mechanism of action appears to be connected with their high affinity for sterols [5], but controversies exist about the precise molecular-level mechanism [6], especially with regard to interaction with cell membranes. The activity of amphotericin B and nystatin was normally attributed to malfunction and/or disruption (leakage) of the lipid bilayer promoted by their binding to the biomembrane sterols [7], inducing pores or channels and disturbing the selective permeability of the membrane [1,2,8–11]. Recent findings have cast doubt on this hypothesis since a modified amphotericin exhibited antifungal activity without being able to form pores or channels [6]. As for natamycin, this formation of pores or channels is not applicable [6,12], and therefore other mechanisms must prevail [11,13,14].

In this study we try to explain the low toxicity of natamycin to mammalian cells, in spite of its affinity to cholesterol, by investigating the interaction with cell membrane models. We use Langmuir monolayers to mimic a cell membrane for two main reasons, namely the ability to probe molecular-level interactions with vibrational spectroscopy and the possibility of controlling the model membrane composition. Understanding the interaction with membranes is crucial because natamycin is a macrolide polyene antifungal with broad spectrum activity, which is only possible if the action involves the membrane. Natamycin was first isolated

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in 1955 from a soil sample in Natal province, South Africa, produced by the bacteria strain named *Streptomyces natalensis*. It is poorly soluble in water, almost insoluble in nonpolar solvents. Natamycin powder is stable in the dark, with no loss of activity, but it is degraded in aqueous suspensions if exposed to UV-light, oxidants and heavy metals [15,16]. When applied to food surfaces, natamycin does not affect the food quality (color, texture and flavor), being safe for consumption because its oral absorption is negligible [16–18]. Due to its low toxicity, natamycin is approved as a natural food additive, can be used topically for treating mycoses [19,20], and is the only topical antifungal approved by the US Food and Drug Administration (FDA) for topical ophthalmic use [20–22].

Macrolide polyene antibiotics have been successfully studied with the Langmuir monolayer technique [10,23–30]. Here we used binary mixtures of phosphatidylcholine (PC) and cholesterol (Chol), which are the most abundant phospholipid and sterol in mammalian membranes [28,31–33], as model membrane, in addition to neat monolayers of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol for comparison.

2. Materials and methods

2.1. Adsorption kinetics and surface pressure isotherms

Polyene antifungal natamycin was supplied by Fluka, cholesterol was purchased from Sigma-Aldrich and synthetic phospholipid dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids. Fig. 1 displays the chemical structure of the three compounds. DPPC was chosen for this study because phosphatidylcholine (PC) is the most abundant component in membranes of mammals, being an important constituent of the alveolar fluid [34]. All reagents were used without further purification. Spreading stock solutions (0.5 mg/mL) for the lipids were made daily by dissolving each compound separately in chloroform, with mixtures being produced in the desired proportions. Aqueous solution/suspension of natamycin is quite stable [15], which is why aqueous formulations are used in therapeutic applications [20–22] and as food additives [16–18]. In ultrapure water (pH = 5.5–6) natamycin molecules are slightly positively charged, since their isoelectric point is at pH = 6.5 [15,35]. Although natamycin solubility in water has been estimated to be 0.03–1.0 g/L [15,35], our preliminary investigations led to irreproducible results (data not shown) when 0.04 g/L was used for the stock solution. This could be associated with aggregation, since a broadening was observed in the UV–vis. bands for natamycin at 0.04 g/L in Fig. S1 in the Supporting information. Therefore, all experiments were performed with 0.02 g/L (black line) for the natamycin stock solution. The subphase employed was made with ultrapure water (pH = 5.5–6) at 21 ± 0.5 °C.

The kinetics of adsorption for natamycin at the air/water interface, which could contain a lipid monolayer, was investigated using a cylindrical Teflon container with a diameter of 4 cm and a volume of 10 mL adapted on a KSV mini trough. Surface pressure was measured with the Wilhelmy method [10]. Aliquots from lipid stock solutions were spread on the water surface, with a volume chosen to reach the desired final pressure. Natamycin was either injected into the subphase or dissolved in the subphase from the start. Surface pressure isotherms for the lipid monolayers were carried out in a mini KSV Langmuir Teflon trough with 250 mL volume. The number of natamycin molecules dissolved in the subphase (0.15 μmol) was about 3 times the number of lipid molecules spread (0.054 μmol) at the air–water interface. This total amount of natamycin (0.4 μg/mL) in the subphase is well below the safe limit established by USA FDA (20 ppm) [35] and it is also about ten times below the minimum inhibitory concentration (MIC) [19–21,47,48]. Since significant oxidation of cholesterol molecules at the interface may occur after 40 min [32,36,37], the isotherm experiments were performed within 40 min of monolayer spreading. A waiting time of 20 min elapsed for chloroform evaporation and monolayer stabilization. The scan rate for the symmetric barrier compression was 30 cm^2/min. At least three isotherms were acquired for each composition to ensure reproducibility, and the curves shown are representative.

2.2. Analysis of isotherms

The mechanical properties of the monolayers were analyzed by calculating the compression modulus C_s^{-1} from the first derivative (1) [10,23–28,31,38,39]

$$C_s^{-1} = -A\left(\frac{\partial \pi}{\partial A}\right)_T \quad (1)$$

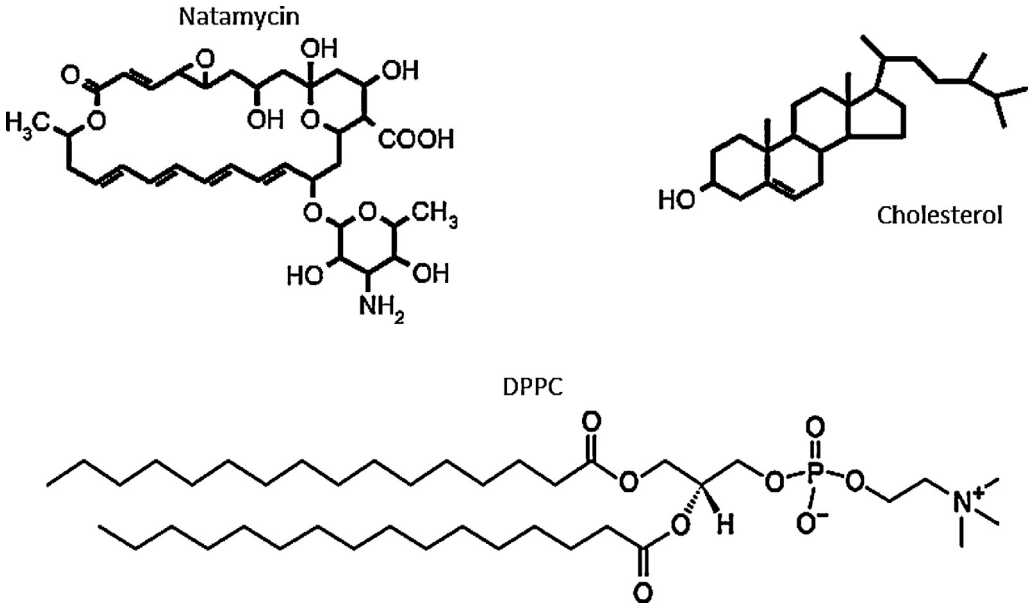

Fig. 1. Chemical structures of the antifungal natamycin, the sterol cholesterol and the phospholipid dipalmitoylphosphatidylcholine (DPPC).